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Thesis

ANALYTICAL REACTIONS OF HISTIDINE

by

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(A. B., Clark University, 1934)

submitted in partial fulfillment of the
requirements for the degree of

Master of Arts

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PREPARATION OF HISTIDINE

An examination into the methods of histidine determination was suggested by Dr. Burnham S. Walker after he and his co-workers attempting to utilize a method of histidine determination in the urine of pregnancy found non-confirmatory results. This study of the "Analytical Reactions of Histidine" includes a preparation of the essential amino acid, histidine, and an investigation into the existing methods of its detection with a probable view of modifying or producing a more practical method.

a. From natural protein

The preparation of histidine from blood corpuscular paste was accomplished according to the details of M. T. Hanke and K. K. Koessler⁹ with but slight modification. Although several newer modes of preparation are evidenced in the literature, methods by L. B. Mendel and H. B. Vickery²⁵, I. A. Smorodinzew³⁵, J. Kapfhammer and H. Spörer¹⁵, H. B. Vickery and C. S. Leavenworth³⁸, etc., only the method of Smorodinzew was also further investigated but this method was abandoned when the results proved of dubious value.

The Hanke-Koessler method consists essentially of hydro-

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lysis of the protein, precipitation of the humin and ferric hydroxide, removal of the tyrosine and leucine, precipitation and the purification of the histidine.

Fresh horse plasma residue* was hydrolyzed with twice its volume of concentrated 37% hydrochloric acid in a weighed 6,000 ml. long necked round bottom Pyrex flask by boiling under reflux for thirty hours over a gas flame. On the addition of the hydrochloric acid the blood cells turned dark brown-black and gathered in clumps. Talcum was added to the contents of the flask to prevent bumping. After completion of the hydrolyzation, the hydrochloric acid was removed by distillation in vacuo at approximately 60° from the same flask. After removal of excess acid the flask and residue was further dried at 112° for two hours to remove the water and acid as completely as possible.

The flask was reweighed and the weight of the residue obtained by difference-900 ml. of the fresh corpuscle paste producing 648 gms. of dried residue. This residue is dissolved in 1850 ml. water, gentle heat

*The horse plasma residue was obtained from the Anti-toxin Laboratories, Forest Hills, Boston, Mass.

3.

applied to cause solution. The solution is then treated with commercial finishing lime producing a reddish brown precipitate. An excess of the lime is added until the solution is completely saturated and the precipitate assumes a homogeneous clay color due to the presence of undissolved lime. This saturation with lime is necessary to insure a complete precipitation of the ferric hydroxide and humin. Care should be taken in the addition of the lime, for much heat is evolved and the effervescence may cause loss of solution unless a suitable roomy vehicle is used for the precipitation. To the cooled solution, 900 ml. of 95% alcohol is now added and subjected to vacuum distillation at 40° until about 1500 ml. of distillate have been collected. This insures complete removal of all the ammonia.

The mixture is then filtered from humin, ferric hydroxide and excess lime on a Büchner funnel. This is a very tedious and painstaking procedure for the humin collects in the pores of the filter and filtration is accomplished only very slowly and with much difficulty. Finally on completion the precipitate is carefully washed with a hot saturated solution of lime. The clear amber-colored filtrate, which contains all the

amino acids as lime salts, is free from iron, if enough lime has been added.

The alkaline solution thus obtained is diluted to 6000 ml., heated and treated with 600 gms. solid anhydrous sodium carbonate*. The solution is agitated until the solid has completely passed into solution. This procedure immediately precipitates the calcium, as the insoluble carbonate, very voluminously. This precipitate is filtered off on the Büchner funnel and washed with a quantity of hot water. The filtrate, which is now freed from calcium, is transferred to two six liter flasks, cooled under the tap, and treated with concentrated 37% hydrochloric acid until the liquid reacts neutral to litmus paper. Glacial acetic acid is then added until effervescence ceases, the solution then being subjected to a distillation in vacuo at 50-60° until the volume has been reduced to about 1500 ml. At this concentration a quantity of sodium chloride together with tyrosine and leucine start to crystallize. The batch of material is placed into the refrigerator for four days thus separating the tyrosine al-

*An equal quantity of carbonate is added in respect to the weight of the dried residue. 600 gms. of carbonate were here only added due to a slight loss of material in the lime treatment by overflow.

most completely, considerable leucine, as well as the remaining sodium chloride. The crystalline precipitate is filtered on the Büchner funnel and washed with ice-cooled water.

The light amber colored filtrate, which contains the histidine, is diluted to 3500 ml. This filtrate is divided into seven equal parts, of 500 ml. portions each. Each portion is transferred to a 4000 ml. beaker and diluted with 1500 ml. water. Further discussion will now be limited to one of these portions. The other six portions are treated in a manner identical to that which will be described for one portion.

It is at this point that deviation from the original procedure takes place. Hanke and Koessler recommend the addition of a mercury salt, mercuric chloride, as the precipitant of the histidine. But due to the expense which would be incurred in the procurement of the necessary sublimate, another precipitant had to be obtained which could be procured in quantity, having a similar chemical action; lead was selected in the form of sugar of lead, lead acetate, as capable of reacting under similar conditions. 350 gms. of solid lead acetate* is then added to the acid liquid and agi-

* 350 gms. of lead acetate is four times the weight of orig-

b.

tated until the solid goes into solution. This process may be hastened somewhat by heating slightly. After complete solution, the liquid is cooled, 70 gms. of solid anhydrous sodium carbonate* is carefully added with stirring to cause solution. This precipitates the lead salt of histidine in the form of a flocculent white solid that settles readily leaving a clear supernatant liquid. A test portion of this liquid should give no immediate precipitate when it is treated with a sodium carbonate solution.

The slightly yellowish supernatant liquid, basic, is removed as completely as possible by means of a glass siphon. Actually this liquid is to be discarded but instead, to a sample portion, a rough qualitative test for histidine was applied with positive results, confirming the suspicions that all the histidine had not been precipitated by the lead but had remained in solution. The manner in which this filtrate was treated will be shortly discussed**.

Continuing, 3000 ml. water is poured into the beaker onto the lead precipitate, the mixture thoroughly agitated,

inal residue that is present in each portion.

*70 gms. of anhydrous sodium carbonate represents 20 gms. of carbonate for every 100 gms. of lead salt.

**This liquid will later be referred to as filtrate A.

the precipitate allowed to settle and the supernatant liquid again drawn off as before and discarded. The lead salt is washed seven times further in this manner. The seven batches of washed and cleaned lead salt so obtained are combined into two six liter flasks. Hydrochloric acid, 37%, is then added to the solid causing effervescence by decomposition of the excess lead which had precipitated as the carbonate, lead carbonate converted into the chloride, which being very insoluble, did not go into solution but remained behind as a white solid. The histidine which was present dissolved in the concentrated acid. The acid solution was then separated from the insoluble lead chloride after the further addition of hydrochloric acid failed to cause effervescence, indicating that the entire carbonate had been decomposed.

The pale yellow filtrate is subjected to saturation with hydrogen sulphide by passing the gas through the acid solution for 24 hours. There appears a reddish precipitate which soon turns to the black of the lead sulphide, this process setting free the histidine from its salt and liberating the lead, precipitated by the hydrogen sulphide. The resulting mixture is filtered on the Büchner funnel and the filtrate, clear and

colorless, except for a little colloidal sulphur, is subjected to vacuum distillation at 60° . Whereas Hanke and Koessler obtained upon this treatment of the removal of the acid a pale yellow, exceedingly stiff gum, a crop of crystals were here obtained which was freed from water and hydrochloric acid as completely as possible by heating in vacuo at 80° for two hours.

The crystals so obtained are dissolved in 100 ml. concentrated hydrochloric acid by heating on the water bath and freed from insoluble sodium chloride. On cooling the crystals of histidine dichloride appear, the process somewhat hastened by placing the mixture in the refrigerator for two days to ensure complete precipitation. The grayish white crystals are filtered and washed with cold concentrated hydrochloric acid and are directly treated for further purification. The entire crop of crude histidine dichloride is dissolved in 35 ml. hot water. 350 ml. hot 95% alcohol is added to the above aqueous solution and this mixture heated on the water bath until the alcohol boils. This procedure causes some inorganic material to precipitate out, such as sodium chloride. While hot, the alcoholic solution is filtered into a 500 ml. beaker.

The first part of the paper is devoted to a general discussion of the problem of the origin of life. It is shown that the problem is not only a scientific one, but also a philosophical one. The scientific aspect of the problem is concerned with the question of how life arose from non-life. The philosophical aspect is concerned with the question of whether life is a necessary part of the universe or whether it is a mere accident.

The second part of the paper is devoted to a discussion of the various theories of the origin of life. It is shown that there are three main theories: the theory of spontaneous generation, the theory of panspermia, and the theory of abiogenesis. The theory of spontaneous generation is the oldest and simplest, but it is also the least plausible. The theory of panspermia is the most plausible, but it is also the most difficult to test. The theory of abiogenesis is the most recent and most complex, but it is also the most promising.

The third part of the paper is devoted to a discussion of the various experiments that have been carried out to test the theories of the origin of life. It is shown that there have been many experiments, but none of them have been conclusive. The experiments have shown that life can arise from non-life under certain conditions, but they have not shown that life can arise from non-life under the conditions of the early earth.

The fourth part of the paper is devoted to a discussion of the various implications of the theories of the origin of life. It is shown that the theories have important implications for our understanding of the universe and for our understanding of life.

The nearly colorless filtrate deposits light grayish plates of histidine dichloride on cooling and is allowed to remain in the refrigerator for 24 hours after which the crystals are filtered. From the mother liquor further crops of crystals may be obtained. These may be further purified until pure white in color by crystallization from hot 95% alcohol. The total yield of pure histidine dichloride thus produced amounts to 2.5 gms. The amount obtained did not coincide with the figures that Hanke and Koessler quote, the greater part assumedly being still in the alkaline liquid which was retained as previously stated.

Filtrate A, the alkaline supernatant liquid referred to above, is acidified with glacial acetic acid and subjected to distillation in vacuo at 80° until completely dry. Evaporation in this manner causes an enormous mass of white crystals to appear. This material was dissolved in the least amount of hot water and a larger volume of hot 95% alcohol added, the mixture brought to a boil and filtered from inorganic salt. This method of extraction removed the total solid present almost completely except for a very small amount of inorganic salt. On cooling, clear colorless prismatic crystals were precipitated. These crystals were filtered from

the mother liquor and further dissolved in hot 95% alcohol. They completely went into solution and re-crystallized on cooling. The crystals were again removed from solution and placed in the drying oven where they underwent a dehydration process similar to loss of water of crystallization, and changed in color from clear colorless to a white amorphous compound which was ground into a powder.

A small sample was heated several hours at 110° to completely ensure dehydration, ground finely and preserved in a weighing bottle over sulphuric acid. This sample when treated for the Pauly histidine test gave a very deep coloration, indicative, it seemed of comparative large amounts of the compound in question. A melting point determination showed that the compound or mixture melts at a temperature about 220° . The powdered material has a strong fishy odor. A Kjeldahl for nitrogen gave a value of about 0.50%. If this nitrogen was contained only in the histidine molecule, it was calculated that there must be between 25-30 grams of histidine in the mixture which would explain the little yield of amino acid from the previous process. That the larger part of this mixture is purely organic may be assumed from the fact that it is completely solu-

ble in 95% alcohol, a characteristic not commonly exhibited by the inorganic salts, and especially uncommon of those salts used in the preparation of the histidine, sodium carbonate, lead acetate, sodium acetate, etc. A qualitative test on a portion dissolved in water, acidified and treated with hydrogen sulphide gave no precipitate, indicative of a negative lead test. Although tyrosine also reacts to the Pauly test for histidine, during the original procedure, the tyrosine was almost completely precipitated in one of the early steps; this fact substantiated by a negative Millon test.

The removal of this "histidine" from the salt mixture is another problem and is yet to be considered.

The alternative method of Smorodinzew for the histidine preparation was started but finally abandoned due to the uncertainty of mode of procedure, reagent addition and production of results. He recommends the boiling of two volumes of blood with one volume concentrated hydrochloric acid for ten hours and the excess acid evaporated, which was accomplished at ordinary atmospheric pressure, no directions given contrarywise. The residue was then neutralized by the addition of a solution of sodium carbonate until only a very

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slight acid reaction is observed. The mixture is filtered from humin, etc., the filtrate made alkaline with sodium carbonate and boiled for the removal of ammonia. The histidine is then precipitated by a solution of mercuric sublimate and filtered from solution. The precipitate is dissolved in a small amount of hydrochloric acid, made alkaline again by sodium carbonate and the amino acid reprecipitated by sublimate. This precipitate now obtained is washed and decomposed with hydrogen sulphide, the mercury precipitating as the sulphide with free histidine in solution. The filtrate from the hydrogen sulphide treatment is concentrated until the histidine crystallizes out. After further recrystallization the histidine is obtained pure. From one kilogram of dried ox blood Smorodinzew obtained 43 grams histidine chloride.

This method was followed to the first precipitation of histidine by mercury. Here a black precipitate was obtained which would not dissolve on further treatment with concentrated hydrochloric acid as directions state. The uncertainty of results are laid to the incomplete directions, of the non-indication of specific amounts of reagents to be added and laxity

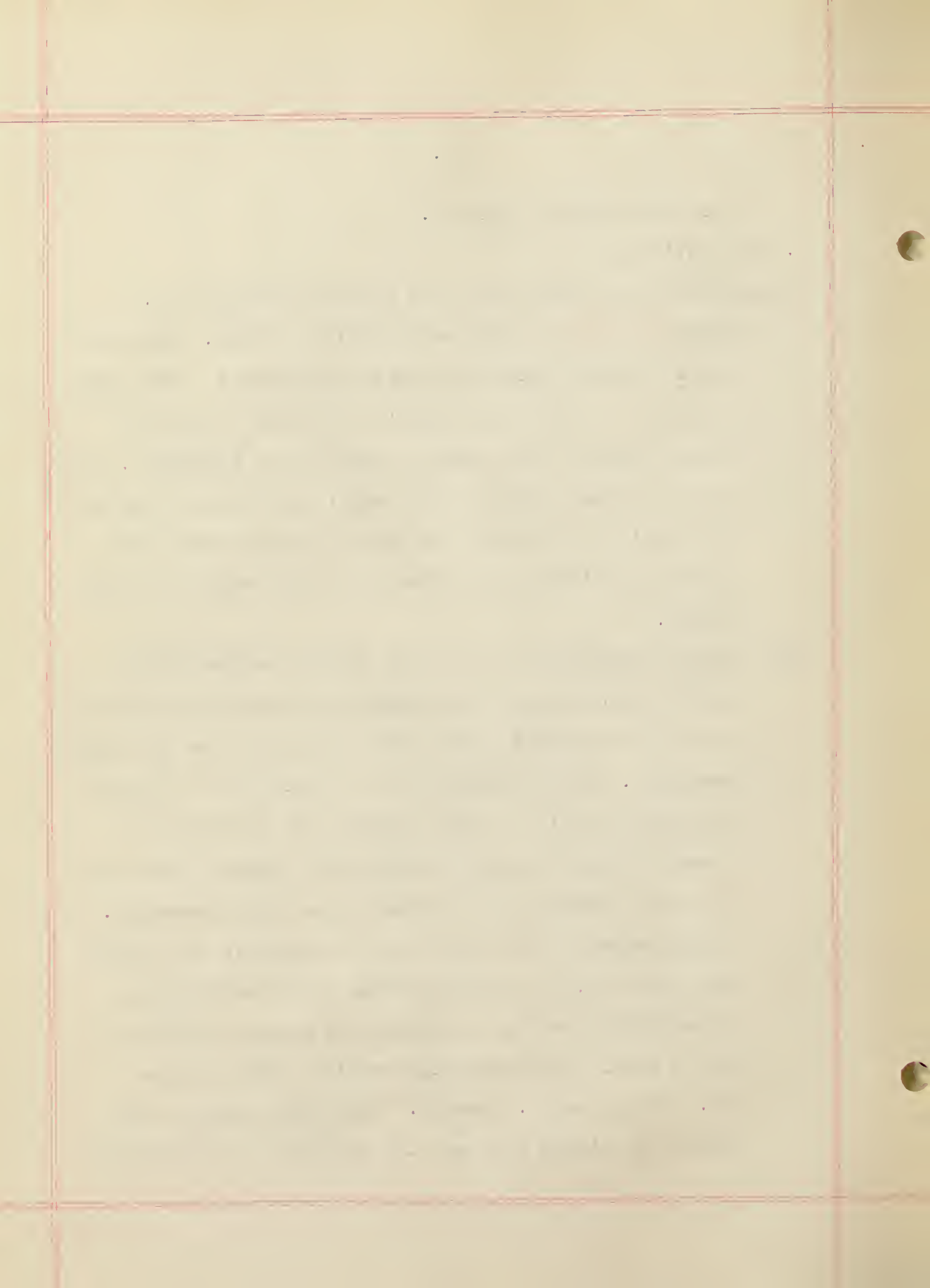
of directions in general.

b. Synthetically

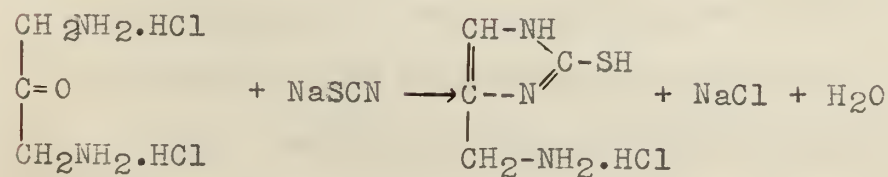
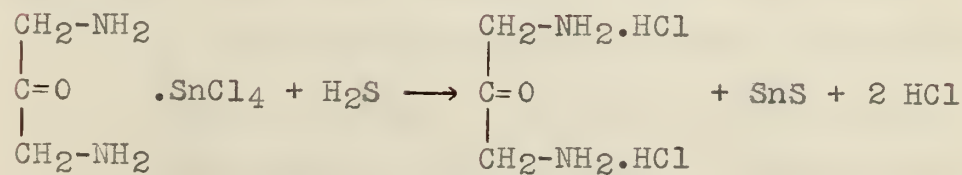
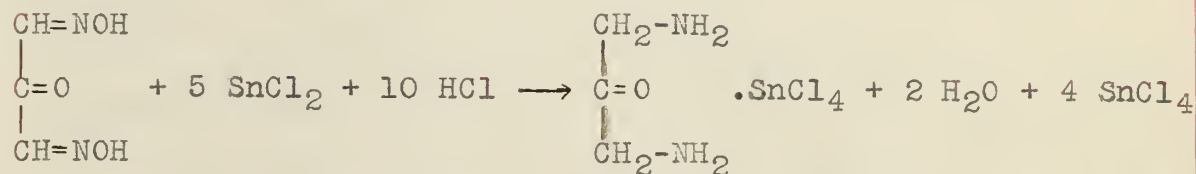
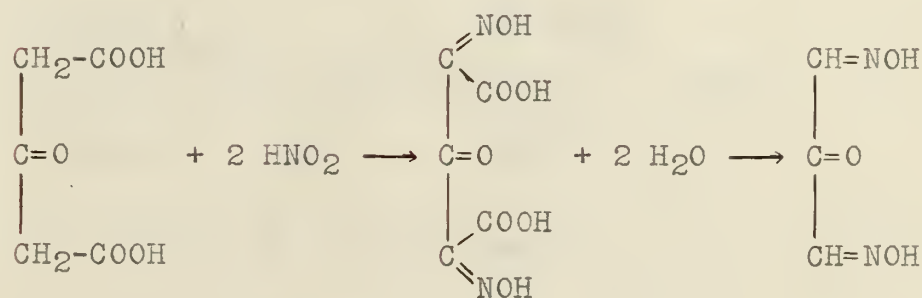
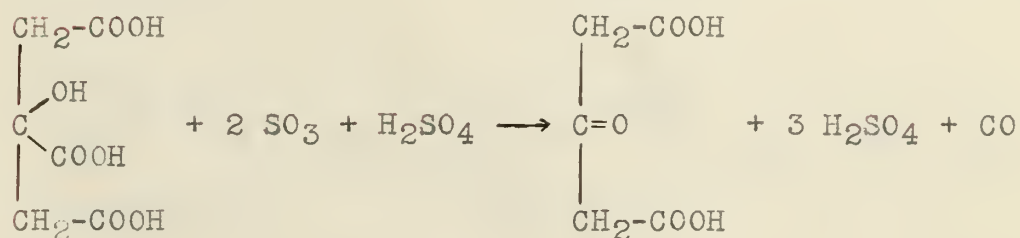
A synthesis of histidine has been accomplished by F. L.

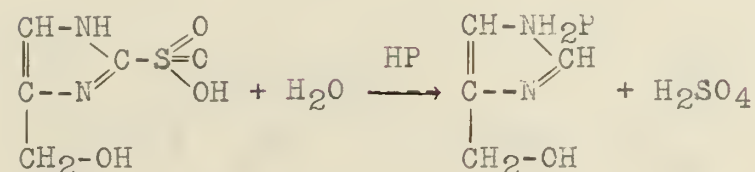
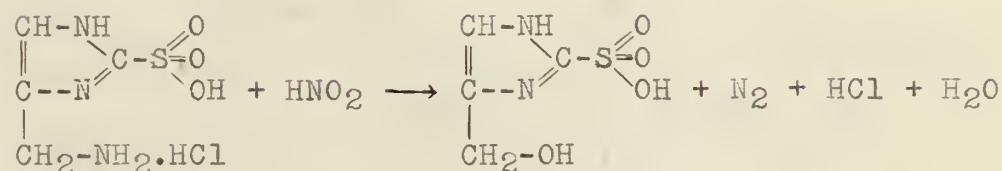
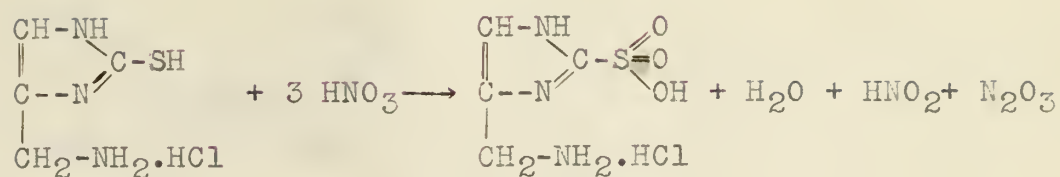
Pyman^{32,33,36} in 1911 and modified in 1916. The early part of this synthesis is best described by Hanke and Koessler¹⁹ who modify Pyman's synthesis in part as they describe the parallel synthesis of histamine. It was following Koessler and Hanke's directions that the synthesis of histidine was to be accomplished with a view to modifying the methods of Pyman after a certain point.

This synthesis as described follows in this manner-citric acid is decomposed with fuming sulphuric acid producing acetone dicarboxylic acid with the liberation of carbon monoxide. This resulting acid is decomposed by means of sodium nitrite to the formation of diisonitroso-acetone which in turn is reduced by stannous chloride with the formation of diaminoacetone chlorostannite. After removal of the tin salt by treatment with hydrogen sulphide, the diaminoacetone dihydrochloride is treated with sodium thiocyanate with the formation of the 2 thiol 4 aminomethylglyoxaline hydrochloride (S. Gabriel and G. Pinkus).⁶ The thiol group is oxidized by nitric acid and the resulting compound pre-

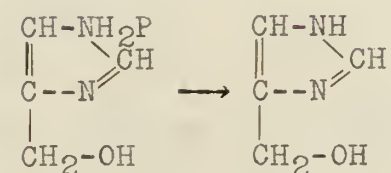


precipitated as the picrate. The picric acid is removed to give free 4 hydroxymethylglyoxaline hydrochloride.





Where HP = picric acid

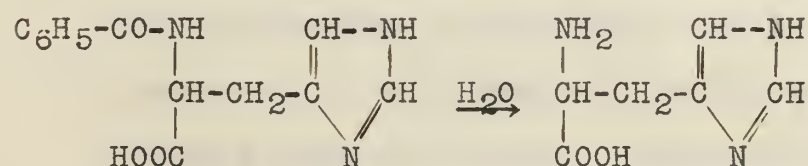
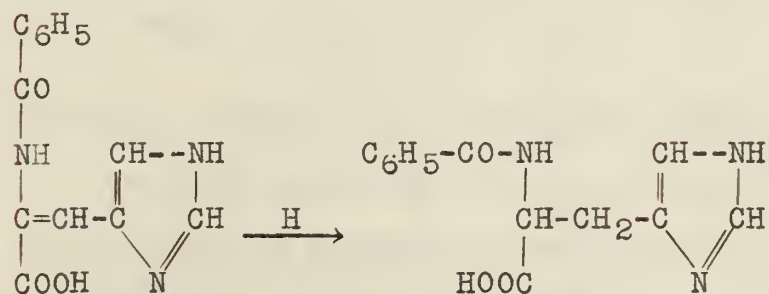
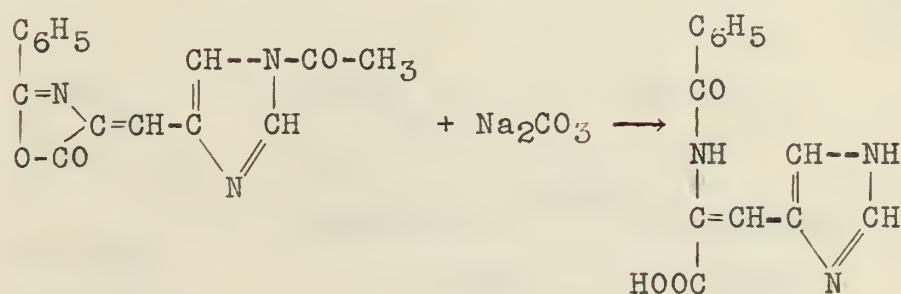
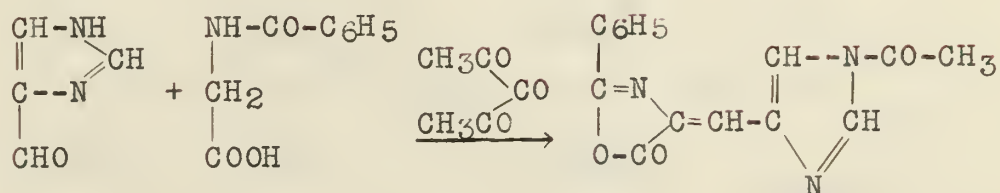
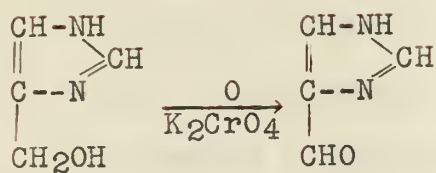


From this point the methods of Pyman are these---

a. the 4 hydroxymethylglyoxaline hydrochloride is oxidized to the 4 glyoxaline formaldehyde, treated with hippuric acid to form a condensation product, 2 phenyl-4 (1 acetyl glyoxaline-4 methylidene) oxazolone, the oxazolone ring ruptured by dilute alkali, reduced to give benzoyl histidine and hydrolyzed to histidine.

The first of these is the
 fact that the system is
 not self-sufficient. It
 requires a constant supply
 of raw materials and
 energy. This is a major
 problem for the system
 as a whole. The second
 problem is the fact that
 the system is not
 flexible. It is unable to
 adapt to changing
 circumstances. This is a
 major problem for the
 system as a whole.

The third problem is the
 fact that the system is
 not sustainable. It is
 unable to maintain itself
 over the long term. This
 is a major problem for
 the system as a whole.
 The fourth problem is the
 fact that the system is
 not equitable. It is
 unable to provide for the
 needs of all its members.
 This is a major problem
 for the system as a whole.

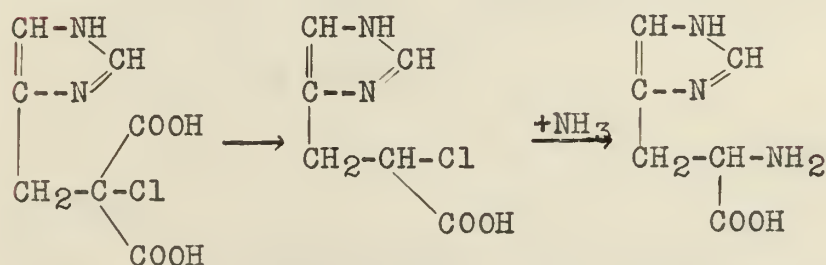
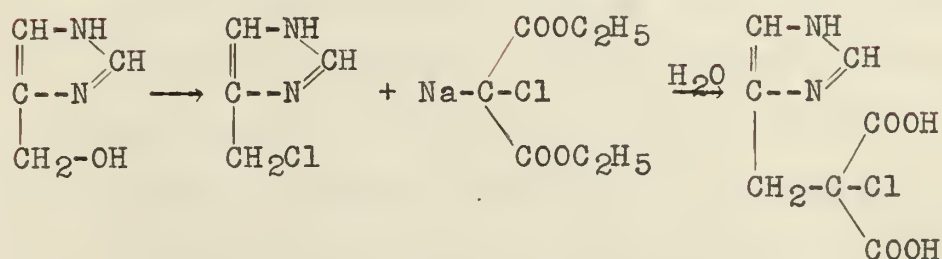


b. The 4 chloromethylglyoxaline is prepared and reacted with ethyl sodiochloromalonate, the resulting product heated for removal of one carboxyl group and

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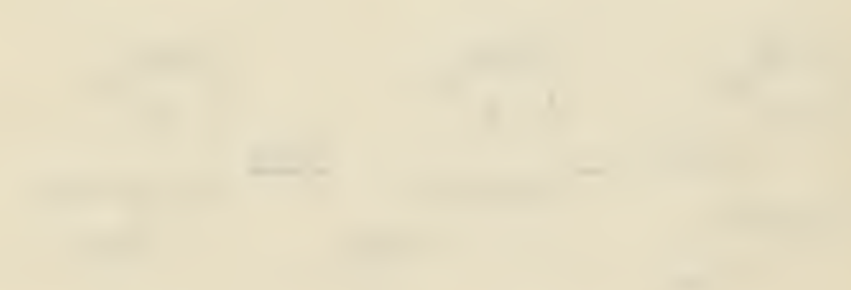
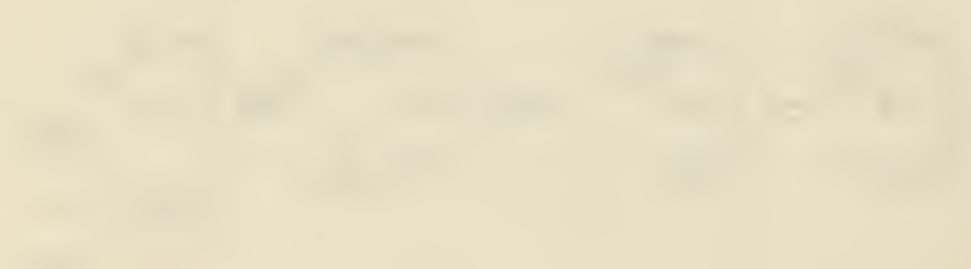
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further treated with ammonia for the replacement of the chloro radical with amine to give the resulting compound histidine.³⁴



In actual procedure the attempts of synthesis following these methods proved to be non-productive. This method of Hanke and Koessler, modifications of methods by H. von Pechmann and K. Wehsarg³¹ and H. von Pechmann³⁰, as well as further modifications of the original by C. K. Ingold and L. C. Nickolls¹² and H. Gilman⁸, were attempted without success. Personal variations of these methods given were also put to use, but from the acetone dicarboxylic acid no further product could be isolated falling in line with the original synthesis. For this obvious reason this train of synthesis was

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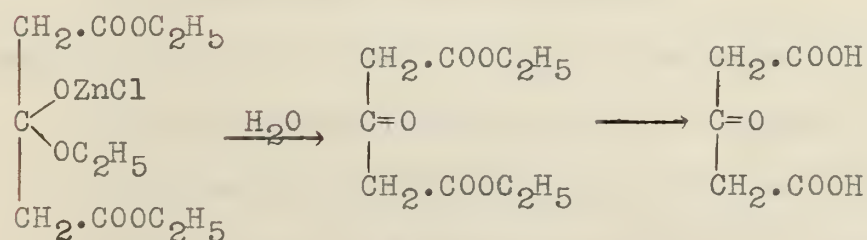
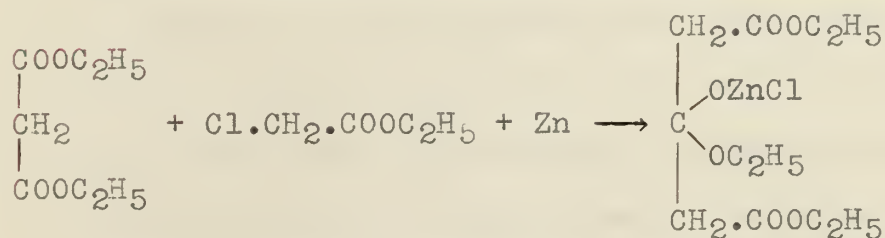


1. The first step in the synthesis of the compound is the reaction of the starting material with the reagent. This reaction is carried out in a round-bottomed flask equipped with a magnetic stirrer and a reflux condenser. The reaction mixture is stirred for 24 hours at room temperature. The progress of the reaction is monitored by thin-layer chromatography (TLC) using a 10% ethyl acetate/hexanes solvent system. The starting material is visualized by spraying the plate with a 1% ceric ammonium molybdate solution in 10% sulfuric acid. The product is isolated by extraction with diethyl ether and dried over anhydrous sodium sulfate. The crude product is then purified by column chromatography on silica gel, eluting with a gradient of ethyl acetate in hexanes. The pure product is obtained as a white solid, mp 105-106°C. The yield is 78%.

2. The second step in the synthesis is the reaction of the intermediate with the reagent. This reaction is carried out in a round-bottomed flask equipped with a magnetic stirrer and a reflux condenser. The reaction mixture is stirred for 24 hours at room temperature. The progress of the reaction is monitored by thin-layer chromatography (TLC) using a 10% ethyl acetate/hexanes solvent system. The starting material is visualized by spraying the plate with a 1% ceric ammonium molybdate solution in 10% sulfuric acid. The product is isolated by extraction with diethyl ether and dried over anhydrous sodium sulfate. The crude product is then purified by column chromatography on silica gel, eluting with a gradient of ethyl acetate in hexanes. The pure product is obtained as a white solid, mp 105-106°C. The yield is 78%.

abandoned.

The preparation of the acetone dicarboxylic acid had also previously been attempted according to the Reformatsky reaction as described by W. T. Lawrence²³, in a parallel reaction. Ethyl malonate in the presence of powdered zinc should combine with ethyl chloroacetate, prepared according to M. Conrad⁴, to form a condensation product which on hydrolysis ought to give the desired dicarboxylic acid, according to the following equations.



Closely following the procedure of Lawrence who describes a synthesis of citric acid from ethyl oxalylacetate by an analagous method, however, none of the desired product could be obtained, only a slight reaction occuring between the reagents.

The next point in discussion was the preparation of diaminoacetone. One method of procedure attempted was in the preparation of dichlorhydrin by the saturation of glycerine with hydrogen chloride then either replacement of the chlorine groups directly by ammonia and oxidizing to diamino acetone, or first by oxidizing and then replacement, W. Markownikoff²⁴. However, the preparation of the dichlorhydrin, although simply described, M. Berthelot¹, H. Hübner and K. Müller¹⁰, etc., results also in the production of numerous isomers, so contaminating the final products that purification results in a loss of the larger percent of product and would render the preparation and further utilization impractical. For these reasons this procedure was also abandoned.

The preparation of the triose, dihydroxyacetone, would perhaps have simplified matters, but such a procedure was practically impossible under existing conditions. The triose is generally prepared by bacterial action, sorbose bacterium, upon glycerine (G. Bertrand)². Here again the end products of the digestion of glycerine or fruit juices by the bacteria are so varied and impure, as well as of doubtful quantity, that no further thought could be given to this mode of attack.

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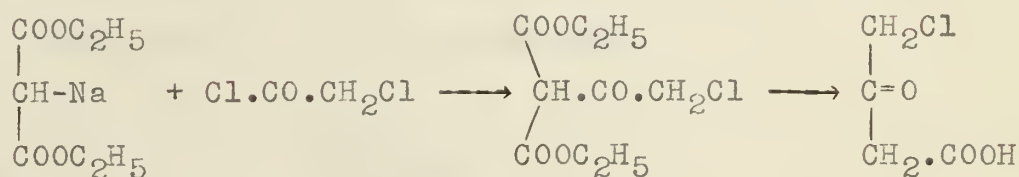
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The reaction of chloracetylchloride with monosodium ethyl malonate was contemplated in a manner such as this---



This reaction is partially theoretical, although a reaction between acetylchloride and monosodium ethyl malonate has been described, A. Michael²⁷ and E. Lang²², and would have been attempted had not the preparation of the chloracetylchloride, as described by A. Michael²⁶ and P. de Wilde⁴⁰, produced such varied results.

Here again as in the preparation of the dichlorhydrin, the impurity of product, quantity and quality of the preparation made it seem worthless to continue.

The most favorable other preparation, which has not unfortunately been hitherto completed is the reaction between methyl iminazole and chloral as described by O. Gerngross⁷. This reaction has not been deeply examined by the author himself, it seems, for he notes two reactions between the reagents depending upon varying external conditions. He is lead to believe that either one or two moles of chloral react with one mole of methyl iminazole and these reactions

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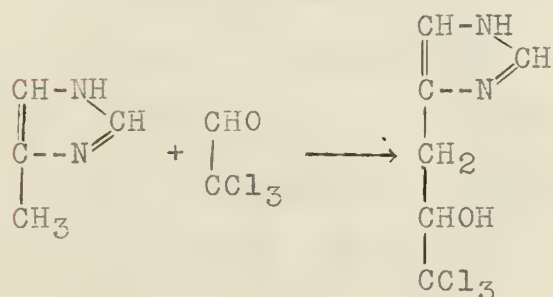
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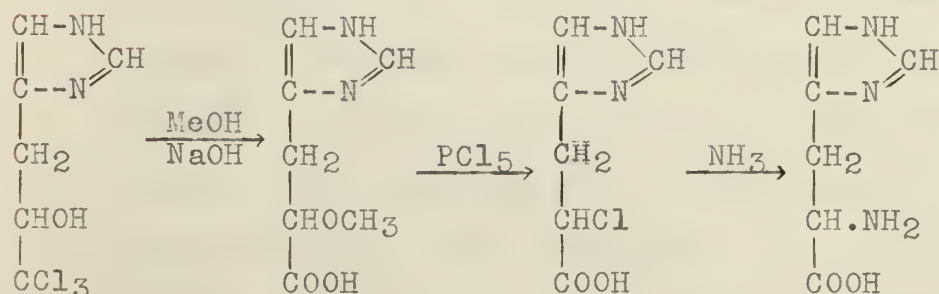
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should be further investigated with a view towards the synthesis of histidine.

The normal reactions, and one which does occur, in part, at least, seem to go in this manner.



This product, he avers, is capable of further change by hydrolysis of the 3 chlorine groups to a carboxyl group, yet he does not seem to have prepared histidine, which was the ultimate aim of the paper, a step which seems logical and easily accomplished---



In an attempt to carry out these procedures, the preparation of methyl iminazole was carried out in accordance with the directions of A. Windaus and F. Knoop⁴¹, with but

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a slight modification which decreases the time of preparation by six weeks. The method as described consists in dissolving zinc hydroxide, washed and dried prepared from one part crystalline zinc sulphate in one and one half parts 25% ammonium hydroxide in a large flask. One part glucose is then added and also dissolved after which a small amount of formaldehyde is then added to increase the rate of reaction. The flask is well stoppered, sealed with wire and well shaken. At this point where Windaus and Knoop recommend allowing this solution to stand at room temperature for six weeks for the completion of reaction, it has been found that the reaction occurs in its entirety if the flask and its contents are kept at a temperature of 48-52° for 24 hours. Color changes occur identically as described in the original literature, turning from yellow through to a deep brown with the separation of a fine yellow brown powder. This precipitate is filtered and is the crude methyl iminazole zinc hydroxide.

The purification follows the method of Windaus and Knoop as modified by Koessler and Hanke¹⁶. The solid crude base is dissolved in a large amount of water acidified with an excess of acetic acid (the base is sol-

uble only in acid solution) and treated by saturation with hydrogen sulphide. This process decomposes the crude methyl iminazole zinc hydroxide by precipitation of the zinc as the sulphide, yellowish due to coloration of the solution. The zinc sulphide is then filtered and the filtrate is distilled in vacuo at 50° ; a gummy brown mass is the result after the water has been entirely removed. This gum is extracted with cold dry acetone and the methyl iminazole precipitated as the oxalate by the addition of a solution of oxalic acid in acetone. The precipitate is recrystallized from 75% acetone and has a pale yellowish gray color. This product is to be further recrystallized from acetone until it has a M. P. of 206° . The free base may be obtained by reversion to the directions of Windaus and Knoop⁴¹.

Whereas Gerngross attempted to study the reaction of the base with chloral, it brought about the speculation whether a similar reaction could occur using glyoxalic acid as the reactant. This procedure would save considerable time in the preparation if the reactivity of the aldehyde group here is similar to that in the chloral molecule, where it is in close contact to the halogen groups.

The first part of the paper discusses the importance of the study and the objectives of the research. It then proceeds to a literature review, followed by a description of the methodology used in the study. The results of the study are presented in the next section, followed by a discussion of the findings and their implications. The paper concludes with a summary of the main points and a list of references.

The study was conducted in a laboratory setting, using a sample of 100 participants. The participants were divided into two groups, each receiving a different treatment. The results of the study showed that the treatment group received the intervention showed significantly better results than the control group. This finding has important implications for the field of research, as it suggests that the intervention may be effective in improving outcomes. The study also identified several limitations, including the small sample size and the lack of a long-term follow-up. Future research should aim to address these limitations and further explore the effectiveness of the intervention.

The study was funded by the National Institutes of Health, and the results were published in the Journal of Health Psychology. The authors would like to thank the participants for their contribution to the study and the research team for their support and assistance throughout the project.

EVALUATION OF EXISTING METHODS OF DETECTION AND DETERMINATION

Of the analytical reactions for the detection of histidine perhaps the Pauly^{28,29} reaction is the most widely known and widely used for quantitative estimation. However as a matter of interest, there is also the Knoop^{20,21} qualitative test with modifications by G. Hunter¹¹ for various existing conditions.

The Knoop reaction consists in acidifying slightly a solution of histidine, adding bromine water solution to excess and boiling to remove excess bromine. This procedure causes the histidine solution to become a brownish red. This procedure has its modifications for deeply colored solutions of various proteins or hydrolysis products, etc., due to the efforts of Hunter.

The Knoop reaction is merely of use as a rough qualitative reaction, but recently R. Kapeller-Adler¹⁴ has brought forward a modified procedure which she has placed on a quantitative basis. The fundamental treatment, that is the bromination of the histidine, is the identical procedure of Knoop but is adapted here by further treatment into quantitative measure.

The black substance which is obtained by the bromination of histidine will dissolve in concentrated ammonium hy-

dioxide with a purple red coloration and in ammonium carbonate with a strong blue violet color.

The reagents are prepared in this manner---

1% bromine solution in 33% acetic acid-5 ml. bromine is dissolved in 500 ml. acetic acid and diluted with 1500 ml. water.

Concentrated ammonium hydroxide, 2 parts, and 1 part 10% ammonium carbonate are mixed to solution.

Standard histidine solution, 1 mg. per 100 ml. water. 100 mg. is dissolved in 2 ml. 10% sulphuric acid and diluted to 100 ml. with water.

To one or two milliliters of standard or unknown histidine solution drop by drop the bromine solution is added until a permanent yellow color appears. After ten minutes 2 ml. of the ammonia mixture is added and the final solution placed into boiling water for five minutes. After cooling, the solution is allowed to stand further for ten minutes, then filled up to 10 ml. volume with 96% alcohol and examined in the colorimeter.

The Pauly reaction takes into account the coupling of a diazo benzene p-sulphonic acid in alkali solution to the histidine molecule, particularly at the amino group; the presence of the iminazole group also couples

the first of the two main groups of the world's population

the white race, which is the most numerous

the black race, which is the most numerous

the yellow race, which is the most numerous

the red race, which is the most numerous

the brown race, which is the most numerous

the pink race, which is the most numerous

the grey race, which is the most numerous

the green race, which is the most numerous

the blue race, which is the most numerous

the purple race, which is the most numerous

the orange race, which is the most numerous

the silver race, which is the most numerous

the gold race, which is the most numerous

the iron race, which is the most numerous

the steel race, which is the most numerous

the copper race, which is the most numerous

the zinc race, which is the most numerous

the lead race, which is the most numerous

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the antimony race, which is the most numerous

the arsenic race, which is the most numerous

the mercury race, which is the most numerous

the bismuth race, which is the most numerous

producing the characteristic color. As Pauly states the reaction, it is to proceed as follows---a fresh solution of diazo benzene p-sulphonic acid is prepared by the addition of sodium nitrite to a solution of sulphanilic acid in dilute hydrochloric acid. The solution to be tested is made strongly alkaline with sodium carbonate and the diazo benzene p-sulphonic acid solution added. A positive test will show a deep cherry red coloration after three minutes which persists on dilution and changes to orange when the solution is made acid. Responses to this test are in the order of concentrations as low as 1:100,000. Only tyrosine and histidine will give this above reaction. Tyrosine gives a yellow coloration however on dilution and a bronze yellow on acidification. The possibility of tyrosine may be excluded if the solution gives a negative Millon reaction. Pauly stated that histidine gave this reaction because it contained the iminazole nucleus; but he states that pyrrol derivatives and nitrogen compounds with similar ring configurations to the iminazole might give the diazo test. He however did not attempt to apply the diazo reaction to iminazole compounds other than histidine.

Although this reaction has been known for a long time as a qualitative test for imidazoles there has been previous to the work of Hanke and Koessler^{16,17,18} but one attempt made to apply it quantitatively. Weisz and Ssobolew³⁹ applied this method for quantitative work on histidine dichloride and found a maximum color reaction with 1 mg. under the conditions specified by them. No attempt was made by the above authors to apply this method to other imidazoles.

This method which Hanke and Koessler propose had the following advantages over that of the above authors.

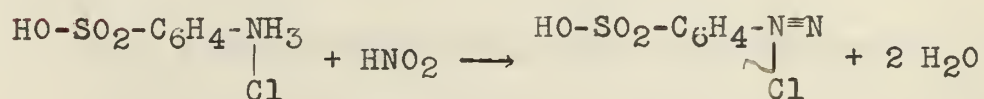
- a. It can be applied directly to practically any imidazole derivative.
 - b. It gives equally good results on mixtures or pure solutions of imidazoles.
 - c. As little as 0.00001 gm. of any of the imidazoles can be estimated with a fair degree of accuracy.
 - d. A laborious series of dilutions is unnecessary.
- The first determination is usually a reliable index of the quantity of imidazole present.

Koessler and Hanke mix the diazo benzene p-sulphonic acid solution first with the alkali sodium carbonate and then proceed to add the imidazole-containing solution to this alkaline liquid to obtain strictly quanti-

The first part of the report is a general introduction to the subject of the study. It discusses the importance of the study and the objectives of the research. The second part of the report is a detailed description of the methodology used in the study. This includes a discussion of the data sources, the sampling method, and the statistical techniques used to analyze the data. The third part of the report is a presentation of the results of the study. This includes a discussion of the findings and their implications. The fourth part of the report is a conclusion and a discussion of the limitations of the study. Finally, the report includes a list of references and an appendix containing additional data and figures.

tative color productions, a procedure opposite to that of Pauly. The necessity for such a change in procedure is readily seen when the chemical reactions involved are considered.

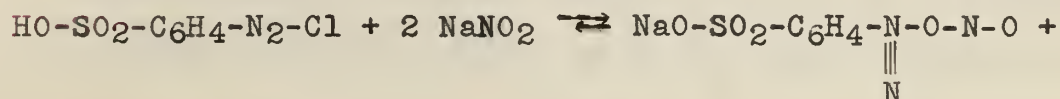
It is generally conceded that the first reaction proceeds according to the following equation.

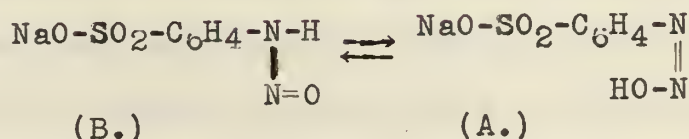
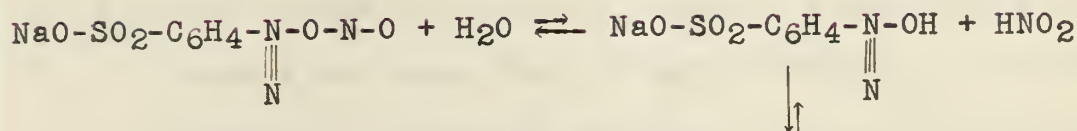
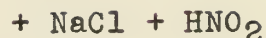


By allowing a mixture containing an excess of hydrochloric and nitrous acids to react for five minutes at 0°, one is assured of a complete conversion of the sulph-anilic acid into a diazonium salt.

After the above reaction has occurred, an excess of sodium nitrite is added to the liquid which brings about an immediate decrease in the concentration of the hydrogen ions with the formation of sodium chloride and nitrous acid.

The series of changes that occur when an excess of sodium nitrite is added to a solution containing the diazonium salt of sulph-anilic acid can be formulated as follows---





The finished solution, after equilibrium has been established, should contain considerable of the compounds (A) and (B) either or both of which are the active coupling agents. It has been found necessary to allow the finished diazo reagent to stand in an ice bath for at least 15 minutes before using to get uniform results.

The above solution contains variable amounts of nitrous acid, variable because some of it escapes in the form of the anhydride whenever the containing flask is opened. If a substance containing an aliphatic amino group, such as histidine, is now added to the above solution, which is the qualitative procedure usually followed, variable amounts of this amino group are replaced by the hydroxy radical. The color production for the colorimetric quantitative procedure depends also on the coupling of the imidazole nucleus with the diazo benzene p-sul-

THE STATE OF

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IN SENATE

January 1, 1900

REPORT

phonic acid reagent.

By a strict adherence to the directions given, which were based upon these theoretical deductions, it has been possible to estimate imidazole derivatives with an accuracy equivalent to that of any colorimetric method.

For a color standard against which to match the colors produced by the reaction, it was of course natural to seek first for stable, chemically allied substances. Both methyl orange and Congo red are chemically related to the colored substances produced with the imidazoles. Combinations of these indicators ought to prove useful as color standards in other cases where the colors produced are yellow to red.

The experimental procedure and preparations of the reagents employed follows---

Stock sulphanilic acid-sulphanilic acid (4.5 gms.) is dissolved in 40 ml. of 37% hydrochloric acid in a 500 ml. volumetric flask and water added to mark.

Stock sodium nitrite-90% sodium nitrite (25 gms.) is dissolved in water and diluted to 500 ml. in a volumetric flask.

Sodium carbonate-J. T. Baker's anhydrous sodium carbonate (5.50 gms.) is dissolved in water and diluted to exactly 500 ml. Other qualities of carbonate (except

Merck's) were found to give yellow colors that were quite different in shade from those produced with the specified salts. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali, Pyrex glass vessels having proved satisfactory.

Stock methyl orange-a good quality of methyl orange (0.5000 gms.) is dissolved in water and diluted to exactly 500 ml. volumetric flask. This solution keeps indefinitely.

Stock Congo red-a similar standard quality of Congo red (2.5000 gms.) is mixed with 50 ml. absolute alcohol in a 500 ml. volumetric flask. Water is then added to the mark. This solution keeps indefinitely.

Standard indicator solutions-for the estimation of histidine, 1 ml. of stock Congo red and 1.1 ml. of stock methyl orange are allowed to flow into 250 ml. water contained in a 500 ml. volumetric flask. Water is then added to the mark. The stock indicator solution must not be mixed in concentrated form. When this is done a rapid interaction seems to occur with destruction of color. When this standard indicator solution is properly prepared and preserved in a tightly stoppered flask, it can be kept with certainty for two weeks.

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It is best to keep this solution in a hard glass vessel, such as Pyrex, because the alkali introduced by the solution of a soft glass changes the color of the indicators enough to give untrustworthy results.

Preparation of diazo benzene p-sulphonic acid solution (the reagent)-1.5 ml. each of the stock sulphanilic acid and sodium nitrite solutions are measured into a 50 ml. volumetric flask. The flask is then immersed in an ice bath for five minutes. Then 6 ml. more of the stock sodium nitrite solution are added and the well mixed solution again allowed to lie in the ice bath for five minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least fifteen minutes after diluting with water. This solution will give perfect results after 24 hours although it is best to prepare a fresh reagent every day.

Stock histidine solution-2.000 gms. of 100% histidine di-chloride is dissolved in water and diluted exactly to 200 ml. A layer of toluene is then added as a preservative.

Standard histidine solution-from the stock solution, 1 ml. is diluted to 100 ml. each ml. of standard solution

containing, then 0.0001 gm. histidine dichloride.

The method used in the general procedure for the estimation of imidazole is illustrated by the following example.

(1-x) ml. of water and 5 ml. of the 1.1 percent sodium carbonate solution are accurately measured into the right hand cylinder of a Duboscq colorimeter, 2 ml. of reagent are measured into a five seconds delivery 2 ml. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. x ml. of the imidazole solution are allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed thoroughly as above. The test cylinder is then transferred to the colorimeter and set at 20 mm. The right hand cylinder which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained. This is the inverse of the process as it is ordinarily carried out where the standard solution is set at some definite value and the test cylinder moved until a match is

obtained.

A maximum color intensity is reached after a gradual development taking about six minutes to reach its maximum density and lasts about two to three minutes. In every case the weaker colors are more stable. The most accurate determinations can be obtained by choosing such an amount of imidazole solution that the standard indicator cylinder has to be set at from 5 to 20 mm. At first a yellow color is obtained which changes rapidly to orange and then more slowly to pink. After reaching the maximum density and remaining there for three minutes, the color changes slowly through orange to yellow.

The color generated by histidine contains slightly more yellow than the standard indicator solution, but the difference in shade is so slight, as they state, that with practice, an intensity comparison can be made with an accuracy of from 0.5 to 3.0%.

A table compounded by Hanke and Koessler using known amounts of histidine and recording the depth of indicator solution shows clearly that the color production is directly proportional to the quantity of histidine dichloride used. This fact can be conveniently represented by the formula

35.

$$I \times 0.000002 = H$$

where I is the depth of the indicator solution in mm. required to match the color in the test cylinder, and H is the number of gms. of histidine dichloride in the test cylinder.

1871
The following is a list of the names of the persons who have been elected to the office of Justice of the Peace for the year 1871, in the County of ...

ATTEMPT AT IMPROVEMENT OR DEVELOPMENT OF ANALYTICAL METHODS

For experimental use a stock solution of histidine was prepared by dissolving 0.5000 gms. of l-histidine dihydrochloride (Eastman Kodak) in 50 ml. distilled water and then adding a layer of xylene to act as a preservative. From this stock solution, a standard solution could be prepared by diluting 1 ml. of stock solution to 100 ml. Each ml. of standard solution then contains 0.0001 gm. of histidine dichloride.

The Knoop reaction was the first to be examined. This consisted in slightly acidifying the histidine solution, adding an excess of bromine water and boiling to remove this excess of bromine. The remaining coloration should be of a brownish red hue, a similar coloration appearing in the case of histamine. This Knoop reaction was repeated time and time again and in the stead of the reported brownish red color, the yellow color of the bromine water was the only discernible reaction color. To ensure a check on the judgment of colors, this test was carried out with a control of pure water also. And here as previously the only color reaction was the straw colored bromine water color so that the difference between the standard con-

trol could not be detected. Such a reaction therefore is of no value even for its qualitative detection of histidine, obviously. Perhaps the nature of the acidifier was to blame. In all the attempts at the production of the Knoop color, a mineral acid was used, either hydrochloric or sulphuric. In the Kapeller-Adler reaction, the acidifier is acetic acid-this combination reacting to bromine to produce a yellow color, which though not the brownish red of Knoop is yet of permanent quality.

For the examination of the modified Pauly reaction of Hanke and Koessler, reagents were prepared exactly as directed previously. Roughly the quantitative reaction consists in the addition of a histidine solution to a freshly prepared solution of diazo benzene p-sulphonic acid in sodium carbonate with the examination of the resulting "cherry red" color under the colorimeter.

Carrying out the mode of reaction as specified, the most important obstacle to the efficient utilization of this method of quantitative examination was the lack of production of the presupposed red color. In its stead was a bright orange, the result of the diazo coupling. Repetition of the method did not alter the facts as they presented themselves. Immediately upon mixing

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the two solutions, reagent and histidine, there appeared a yellow color which after changing to orange persisted in intensity throughout, with a fading after a maximum has been reached. No trace of pink could be seen in the reacting solution, a discrepancy which was inexplicable.

Directly hinging upon this lack of red color formation was the difficulty in matching with the color standard. The color standard is of red cherry hue, similar to the supposed diazo reaction coloration of histidine. This then led to the matching of colors in the colorimetric work by intensity of light rather than by colors. Although far more accurate results are to be obtained by comparisons from intensity of light, this wide color range caused much difficulty especially to the unskilled. However in all attempts which were made on the comparisons, it was possible to obtain a check on the results of Koessler and Hanke and agreement to their formula, $I \times 0.000002 = H$, was obtained. Since in this particular case a known amount of histidine was used in the experiment a check both ways could be obtained by replacement of the two unknowns in the above equation or by comparison to the table of Koessler and Hanke which is derived from essentially

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the same procedure.

However, to afford greater facility in the reading of the colorimeter it probably would be of more advantage to search for a color standard which more closely approximates the actual color formation of the diazo test. To obtain such color standards it must be repeated that it is necessary to find stable, chemically allied substances-related to the colored substances formed in the diazo reaction for imidazoles in chemical structure and action. Since the estimation of amount of imidazole is possible with the methyl orange-Congo red mixture, it would not be a too difficult procedure to recheck the new color standard against the methyl orange-Congo red and by use of suitable constants it could be directly applied to the unknown iminazole solutions.

Rather than search for a new dye color standard, futile, considering the wide range of colors, it was recommended that the color standard be composed of the maximum color produced by the diazo reactions from a known definite amount of histidine. This idea was followed up and examination comparing the colors produced by varying amounts of histidine against a standard containing 0.0001 gm. of histidine continued.

After standardizing the colorimeter, by having both cylinders

contain 0.0001 gm. each, both set at 20 mm., the experimental procedure was started. The unknown solution was placed in the left hand cylinder and set at 20 mm. and the known amount, 0.0001 gm., placed in the right hand cylinder and adjusted constantly until the maximum reading has been obtained, the identical procedure of Koessler and Hanke. The unknown solution as has here been represented, actually contains a known amount of the histidine so that a correlation might be drawn from the readings recorded. A table of several readings as observed follows---

L. at 20 mm.	R. with 0.0001 gm.
0.00005 gms.	11.7 mm.
0.00001 gm.	2.3 mm.
0.00008 gms.	18.4 mm.
0.00004 gms.	10.0 mm.
0.00006 gms.	13.5 mm.

Using the Koessler-Hanke formula, $I \times 0.000002 = H$, substituting the experimental amounts of histidine used, we solve for I. The actual mm. deviation divided by this number produces a rather fair constant value throughout.

gms. histidine	I	$\frac{H}{0.000002}$	$\frac{I}{\text{mm. deviation}}$	K	average K
0.00005 gms.	25.0		2.14		
0.00001 gm.	5.0		2.17		
0.00008 gms.	40.0		2.17		2.14
0.00004 gms.	20.0		2.00		
0.00006 gms.	30.0		2.22		

This would seem to indicate, then, that a definite correlation may be drawn from the Hanke-Koessler formula and the recordings above obtained. Since the values of these constants agree so well, by proper placement of the average constant value, we obtain the formula, $(I \times 0.000002)(K)=H$, which yields the actual amounts of histidine used when solved, substituting for I.

Several precautions have to be taken, however, which hinder the practicability of this correlation. For each determination, a new color standard has to be prepared, for the color fades slightly in shade, which allows discrepancies if speed is not exercised. The maximum color is not produced until at least two minutes, persists for about four, and then fades. Again, this necessity for changing the standard at each determination, permits the possibility for mechanical errors, such as the failure to have the standard contain the exact amount, 0.0001 gm., of histidine in each determination.

However, a disadvantage inherent in this diazo reaction is its non-specificity. This reaction will occur for other iminazole nuclear compounds, amino acids and their derivatives, as well as pyrrol and nitrogen ring

configurations similar to the imidazole ring, to quote Pauly. Tyrosine gives the identical color reaction, except that on dilution the tyrosine color produced is yellow, turning from a hypothetical red and becoming a bronze color on acidification; whereas the red color of the histidine reaction suffers no color change on dilution but turns orange on acidification.

Inouye¹³ has observed that the color reaction does become more specific if benzylation of the histidine was accomplished by the Shotten-Bauman reaction. This deals with the action of benzoyl chloride in an alkaline solution, sodium carbonate, upon histidine, forming a mono benzyolated compound, still capable of coupling to the diazo reagent of the Pauly reaction. Tyrosine on similar treatment forms a dibenzoyl derivative which is non reactive to the diazo benzene p-sulphonic acid. Histamine has both the imidazole ring ruptured and forms also the tribenzoyl compound, incapable of coupling. Speaking of the protein hydrolytic products, Inouye states that the benzoyl chloride and sodium carbonate will convert all other compounds except histidine into derivatives which are non reactive to the diazo benzene p-sulphonic acid and fail to give the color reaction. This would seem

to indicate that materials such as the base, iminazole, do not react with the benzoylating mixture, for they are not commonly found in the protein degradation products-and it is a matter of speculation of the free iminazole base is rendered capable of non combination with the active diazo reagent. Inouye specifically states, however that the benzoyl derivative of histamine is non-coupling. An excess of the benzoyl reagent completely hinders the reaction bringing about another point necessary for correction. There is then a need of further effort on this particular line to create the complete specificity of histidine to the color reaction, either by formation of histidine derivatives which only are capable of combination with the diazo reagent or the development of a reagent which produces a coloration specific to histidine alone.

The method of Kapeller-Adler has been developed primarily in the examination of histidine in the urine. As far as she states, the color reactions due to the addition of the reagents specified are identical, although a check on the colorimetric values has not been attempted.

Recently there has been an out-cropping of methods of histidine determinations on urine, each modifications of the original Pauly but especially adapted to the work

of the investigator, such as those of K. Suzuki and Y. Kaishio³⁷ and O. Fürth and E. H. Majer⁵.

The author wishes to acknowledge the value that C. H. Best and E. W. McHenry³ have proven to be in their paper, "Histamine", which was used as key reference.

SUMMARY

A preparation of histidine was undertaken in order to study the "Analytical Reactions of Histidine". Synthetically it was not possible to prepare the essential amino acid although several methods at the present writing are still under observation. A quantity of histidine, however, was obtained from the hydrolysis products of the natural protein, blood haemoglobin, following the modified procedure of Hanke and Koessler.

The analytical methods of histidine determination of such investigators as Hanke and Koessler, Knoop, Kapeller-Adler, etc., were examined and their qualifications noted.

It was found possible, by proceeding along a new line of attack, colorimetric comparison against a color standard containing a known amount of histidine coupled with diazo benzene p-sulphonic acid, to modify the Hanke-Koessler formula, $I \times 0.000002 = H$, to suit these conditions. Placing the unknown in the left hand cylinder and setting at 20 mm. the reading was obtained with the standard in the right hand cylinder. A correlation then drawn, $(I \times 0.000002)(K) = H$, where K, a constant, equals 2.14, seemed to hold for all readings recorded.

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THEORY

The first part of the paper is devoted to the study of the
general properties of the solutions of the system of
equations (1) and (2). It is shown that the solutions
are unique and that they depend continuously on the
data of the problem. The second part is devoted to the
study of the asymptotic behavior of the solutions as
the parameter ϵ tends to zero. It is shown that the
solutions converge to the solutions of the limiting
problem (3) and (4). The third part is devoted to the
study of the numerical solution of the problem. It is
shown that the numerical solution converges to the
exact solution as the number of nodes increases.

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